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The objective of this project is to identify and map genes capable of restoring senescence to immortal breast cancer cells by functional complementation. The methods used to achieve this goal are cell X cell hybridizations and microcell fusions between the tumor and normal cells containing *gpt* tagged single normal human chromosomes.

Cell X cell hybridizations between breast tumor cells and normal cell lines containing *gpt* tagged human chromosomes 2, 3, 6 and 9 resulted in senesced hybrid clones whereas hybrid clones containing human chromosome 4 did not senesce. Introduction of individual *gpt* tagged normal human chromosomes 6, 9 and 16, by using microcell mediated chromosome transfer technique, showed characteristic morphological and growth properties typical to senescence. In contrast, normal human chromosome 13 did not senesce the breast tumor cells. These results indicate the presence of senescence genes on human chromosome 2, 3, 6, 9 and 16. Continuous cultivation of the senescent hybrids in selection yields revertant clones which retain portion of the introduced chromosome containing the selectable marker but have lost the senescence gene. Revertant clones obtained from the chromosome 16 induced senescent clones were analyzed for the presence of previously mapped chromosome 16 specific markers. Results from these experiments localized the sennescence gene on chromosome 16 to a 2-3 cM region at 16q24.3. Work to clone the senescence gene on chromosome 16q24.3 is in progress.

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Deepthi. E. Reddy 11/25/97

PI - Signature Date

Table of Contents

Deepthi E. Reddy
187-74-0476

Annual Report

Title: Identification and localization of genes which restore senescence in breast cancer cells

P.I. : Deepthi E. Reddy

Introduction

Normal mammalian cells exhibit a limited proliferative potential (1,2,3). At the end of their replicative life span, these cells senescence, becoming enlarged and flattened, and cease to divide (4). In contrast to normal cells, breast and other tumor cells can multiply indefinitely, having escaped senescence as a result of alterations in critical genes (5). Escape from senescence thus represents an important step in tumor progression. Since senescence genes are involved in the negative growth regulation, they are regarded as a class of tumor suppressor genes which induce terminal growth inhibition (6). When immortal tumor cells were fused with normal cells, the immortal growth phenotype was suppressed and characteristic senescence features were evident in the hybrid population (6-10). These data suggest that, like other tumor suppressor genes senescence genes exert a dominant growth-curtailing effect which is overcome only upon inactivation of both alleles (11). Allelic alterations which lead to tumor formation are identified using restriction length polymorphisms and loss of heterozygosity (LOH) studies. These studies suggest the presence of tumor suppressor genes involved in breast cancer on human chromosomes 1, 3, 4, 6, 11, 13, 16, 17, 18, 19 and 20 (12-16). Normal chromosomes 4, 6, 11, 13 and 17 were individually introduced by means of microcell fusion into breast cancer cells. Results from these studies indicated that the transfer of the normal human chromosomes into these tumor cells induced tumor suppression (17-21). Interestingly, chromosome 6 arrested tumor cell growth by inducing senescence in the tumor cells (19). The presence of a senescence gene on chromosome 6 was further confirmed in our lab. In addition, chromosome 6 was shown to induce senescence in not only breast tumor cells but also in SV40 transformed cells (20) and in ovarian carcinoma cells (21). This senescence gene was further localized to 6q 14-21 (21).

The objective of this project is to identify and map genes capable of restoring senescence to immortal breast cancer cells by functional complementation. Normal human chromosomes tagged with a selectable marker will be introduced into immortal breast cancer cell lines by means of the microcell fusion and cell X cell hybridization. Hybrid clones will be examined for morphologic changes and alteration of growth properties indicative of senescence. Thus, chromosomes capable of restoring senescence to immortal breast cancer cells will be identified. Continuous cultivation of senescent cells in selection medium yields revertant clones which retain the selection marker tagged chromosome but have lost the region that is required for the induction of senescence. These revertant clones will be analyzed for the presence of previously mapped donor chromosome specific markers, facilitating the localization of genes which restore senescence to human and rat breast cancer cells. The biological materials generated during these experiments will provide information to clone the relevant senescence genes.

Deepthi E. Reddy
187-74-0476

Hypothesis/purpose:

Since normal human cells senesce at the end of their replicative life span, immortal breast tumor cells are thought to have escaped this block to proliferation during tumor progression. Introduction of normal, intact individual chromosomes carrying the senescence genes into these tumor cells is expected to arrest their growth and restor senescence. Thus, functional complementation will definitely identify chromosomes carrying senescence genes. Further localization of these genes will aid in their cloning and lead to a better understanding of senescence pathways and their role in breast tumor formation.

Background of previous work:

The monochromosomal hybrid library developed in Dr. Athwal's laboratory contains individual human chromosomes tagged with a selectable marker, *gpt* or *neo,* and present in a mouse A9 background. Preliminary work done by me as a graduate student in Dr. Athwal's laboratory involved the mapping of the senescence gene on human chromosome 6 and the transfer of normal human chromosomes by microcell fusion into breast cancer cells in collaboration with Dr. Kaur, who is an assistant professor at the Fels Institute. Experimental details and results of this work are described below.

Transfer of individual human chromosomes into breast cancer cells

a) Human breast cancer cells: Normal human chromosomes 6, 9, 13, 16, 17 and 20 were introduced into MCF-7 and SKBR3 (Table1) using the microcell mediated chromosome transfer (MMCT) technique. Microcell hybrids of these breast cancer cells were examined for changes in morphology and growth properties.

These experiments revealed that introduction of human chromosomes 6, 9, 16, 17 and 20 into immortal human breast cancer cells led to reduced division potential and restoration of cellular senescence. Chromosome transfer colonies generally multiplied for 4-7 population doublings and then ceased to divide. These cells displayed longer division times and became enlarged and flattened as compared to the parental immortal cells. In contrast, colonies bearing the human chromosome 13 exhibited no discernable morphological alterations or decrease in growth rate characteristic to senescence. Presence of the donor chromosomes in microcell hybrids was confirmed by PCR analysis for *gpt* or *neo* sequences.

The cultivation of senescing microcell hybrids bearing human chromosomes 6, 9, 16, 17 and 20 in non-selective medium gave rise to two phenotypically distinct types of colonies, senescing and immortal. These colonies were isolated and analyzed for growth properties and the presence of the introduced chromosome. The introduced chromosome was present in senescing but not in immortal clones. These results confirmed that these chromosomes carry genes which can restore senescence to breast cancer cells.

b) Rat breast cancer cells: In another set of experiments, human chromosomes 6, 9, 13, 16, 17 and 20 were introduced into NMU, a rat mammary tumor cell line. Interestingly, the results obtained with NMU cells were identical to the results with human breast cancer cell lines. Human chromosomes 6, 9, 16, 17 and 20 restored senescence in NMU cells while chromosome 13 did not.

Localization of senescence gene on human chromosome 6

Upon continuous cultivation of the senescent hybrid clones yields revertant clones which have the introduced chromosome but have lost the region(s) of the chromosome that is required for senescence. Analysis of the revertants from human and rat chromosome 6 hybrids with chromosome 6 specific markers mapped this senescence gene to 6q 14-21. This gene was shown in our lab to also restore senescence to ovarian cancer cells (21) and SV40 transformed cells (20).

Aims of the project :

Aim 1: Identification of cell senescence genes by functional complementation in immortal breast tumor cell lines.

Aim 2: Regional localization of senescence genes.

Experimental Methods:

The methods used to identify cell senescence genes by functional complementation in immortal breast tumor cells are cell X cell fusions and microcell fusions. The experiments performed using these experimental methods are described below.

Cell X Cell Fusions

The human breast cancer cell line, MCF-7, and the rat mammary tumor cell line, LA7, were marked with *neo* so that they can grow in medium containing G418 (400 μg/ml). Both these cell lines were fused individually with different normal diploid human cell lines carrying *gpt* integrated into human chromosomes 2, 3, 4 & 7, 6, and 9. Since the breast cancer cell lines are marked by a *neo* marker and the introduced human chromosomes are tagged with a *gpt* marker, the hybrid cells were selected in medium containing MX and G418. Hybrids were allowed to grow in selection medium for more than three months.

A hybrid between an immortal and a normal diploid cell line initially contains the total chromosome content of the two parental cell lines. Since senescence is a dominant phenotype, hybrid cells are expected to display characteristics of the normal cells. From prior experience, it is anticipated that a hybrid which is otherwise destined to senesce can propagate for at least 20-50 population doublings. During this period, as cells multiply, chromosomes which do not carry a selectable marker can segregate at random. If genes required for senescence are present on chromosomes which are eliminated by segregation, colonies of immortal cells will appear in the senescing population while cells are cultured in MX+G418 medium. On the other hand, if a senescence gene is present on the chromosome that also carries the *gpt* marker, it will be retained in all living hybrid cells. In such a situation, the entire hybrid cell population will be senescent. Presence of the senescence gene on the tagged chromosome was verified by concordant loss of the tagged chromosome in non-selective medium.

Microcell mediated chromosome transfer (MMCT) into immortal cell lines

Micronuclei containing one or two chromosomes were generated from a monochromosomal hybrid containing a single normal human chromosome tagged with the *gpt* marker in a mouse cell line, A9. These micronuclei were fused with MCF-7 and SKBR-3, two human breast cancer cell lines, and NMU and LA7, two rat mammary tumor cell lines,.

The hybrid cells were grown in a selection medium containing MX. The resulting hybrid clones were analyzed for growth characteristics and morphological changes. When the transferred human chromosome contained a senescence gene, then the hybrid cells exhibited senescence-associated-characteristics, namely, enlarged and vacuolated cells which cease to proliferate after a certain limited number of population doublings.

Transfer of intact normal human chromosomes 13 and 16 and fragments of human chromosome 16 containing the region 16 q22-qter into the breast tumor cell lines was performed.

Results and Discussion:

Cell X Cell fusions: All rat and human hybrids with normal cell lines containing tagged human chromosomes 2, 3, 6 and 9 senesced indicating the presence of senescence gene(s) on these chromosomes. Human chromosomes 4 and 7 did not have any effect on the cell growth or the morphology of mammary tumor cells from both human and rat origin (Table 2).

One of the difficulties which were encountered during these experiments was as follows. One of the pitfalls that was described in the original proposal was that the segregation of untagged chromosomes in human X human cell hybrids may longer than the remaining proliferative life of the hybrid and revertant clones may not be visible before the death of the hybrid. We had proposed that this pitfall will be overcome by a) repeating the experiments where immortal revertants do not appear, b) using rodent X human fusions as a positive control, and, c) transferring the chromosome by MMCT experiments into human cells to confirm the data from a) and b) above. During the course of the experiments, we found this method to be feasible but time consuming. Furthermore, it was found that MMCT experiments were less time-consuming even when repeated to confirm results, avoid erroneous interpretation which are caused by chromosomal breaks and generate immortal clones for mapping experiments. Results from these experiments were also easy to interpret. Thus it would be of an advantage to the project to use MMCT as means of identifying senescence genes in future experiments.

MMCT experiments: Transfer of human chromosome 16 into all four breast tumor cells resulted in senescence while chromosome 13 did not induce senescence in these cells. These results indicate the presence of a senescence gene(s) on human chromosome 16.

LOH on human chromosome 16 has been found in breast cancer in several different studies. Chromosome 16 is predicted to contain tumor suppressor genes in the regions 16q22-qter, 16q22 and 16q24-qter based on LOH studies (22). Since cellular senescence is considered to be a mechanism for tumor suppression, the senescence gene could be present in these regions. In order to develop a project for my thesis work, I decided to further investigate the possibility of the

presence of the senescence gene on human chromosome 16 in this region (16 q22-qter).

A sub-monochromosomal hybrid library created in Dr. Athwal's laboratory contains fragments of human chromosome 16 tagged with a *gpt* marker. PCR and cytogenetic analysis on these monochromosomal hybrids revealed several fragments of chromosome 16 carrying the region 16q 22-qter (Fig. 1). Two hybrid clones containing human chromosome 16q 22-qter (16AN3-1) and chromosome 16q 23-qter (16AN2-2) were chosen for subsequent microcell fusions. The two chromosomal fragments carrying a *gpt* marker were transferred into all four breast cancer cells by MMCT experiments. Both fragments induced senescence in these cell lines (Fig. 2, Table3). These results confirmed the presence of a senescence(s) gene in the region 16 q23-qter.

Localization of the Senescence gene on chromosome 16 by deletion mapping: Continuous cultivation of the senescent cells in the selection medium gave rise to rare immortal cells from the senescent population. These immortal cells still contain the introduced *gpt* tagged chromosome but have lost the region of the chromosome important for inducing senescence. Analysis of the human and rat immortal revertant clones by PCR with 105 previously mapped chromosome 16 markers was performed to identify the region of the chromosome that is lost in these cells. PCR analysis on 35 immortal rat revertants and 5 human revertants localized the region of the senescence gene to a 2-3 CM region on human chromosome 16 q24.3. Fig. 3 and Fig. 4 show the PCR data with some selected markers from the chromosomal region 16q 23-24. The manuscript describing these results is in preparation and will be submitted for publication by the end of this year.

Summary:

Cell X cell fusions between the human breast cancer cell line, MCF-7 and rat mammary tumor cell line, LA7, with normal diploid cell lines containing gpt tagged chromosomes 2, 3, 4, 6 and 9 were performed. Hybrids between the tumor cells and the normal cells containing gpt tagged chromosomes 2,3, 6 and 9 showed growth and morphological features typical to senescence while chromosome 4 hybrids did not show these characteristic features in both the human and the rat tumor cell lines. These results indicate the presence of a senescence gene(s) on human chromosomes 2, 3, 6 and 9.

MMCT experiments were performed with monochromosomal hybrids containing human chromosomes 13 and 16 into the human breast tumor cell lines, MCF-7 and SKBR-3 and the rat tumor cells, LA-7 and NMU. Introduction of human chromosome 16 induced senescence in all four tumor cell lines whereas the introduction of human chromosome 13 did not induce senescence in these tumor cell lines. These results indicated the presence of a senescence gene(s) on human chromosome 16. Furthermore, introduction of fragments of gpt tagged human chromosome 16 containing the region 16 q22-qter and 16 q23-qter also induced senescence in these cell lines, thus localizing the senescence gene(s) to human chromosome 16 q23-qter. PCR analysis of human and rat revertant clones from the senescent hybrids, further localized the senescence gene to a 2-3 CM region on human chromosome 16 q24.3.

Future Goals:

1) Experiments towards cloning of the senescence gene mapped to chromosome 16q 24.3 - YACs spanning the 2-3 CM region will be obtained commercially. These YACs will be retrofitted with a selectable marker and introduced individually into the breast tumor cells. The YACs containing the senescence gene will be identified by functional complementation.

2) The MMCT experiments involving the transfer of human chromosome 20 into the breast cancer cells will be repeated to confirm the initial observations. In addition introduction of human chromosomes 2, 3, 4 and 11 into the breast tumor cells will be done to confirm the results from cell X cell fusion studies.

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Table 1: Human chromosomes transferred into human and rat mammry tumor cell lines

Cell line	Human chromosomes introduced	Number of colonies	Senescent colonies	Length of Proliferation (months)
Human				
MCF-7	6	6	2	1-2
	9	3	3	1-2
	13	5	0	immortal
	16	2	2	<1
	17	2	2	<1
	20	1	1	<1
SKBR-3	6	2	2	<1
	9	3	3	1-2
	13	4	0	immortal
	16	8	8	1
	20	3	3	<1
Rat				
NMU	6	3	3	<1
	9	3	3	1-2
	13	3	0	immortal
	16	2	2	<1
	17	3	3	<1
	20	1	1	<1

Table 2: Normal cells carrying human chromosomes fused with human and rat mammary tumor cell lines

Cell line	Human chromosome introduced	Phenotype	Length of Proliferation (months)
Human			
MCF-7	2	Senescent	<3
	3	Senescent	<3
	4,7	Immortal	>3
	6	Senescent	<3
	9	Senescent	<3
Rat			
LA7	2	Senescent	<3
	3	Senescent	<3
	4,7	Immortal	>3
	6	Senescent	<3
	9	Senescent	<3

Table 3: Human Chromosomes Transferred into Human Breast Cancer and Rat Mammary Tumor Cell Lines

Cell Line	Human Chromosome	No. of Colonies	Senescent Colonies	Length of Proliferation (months)
Human				
SKBR-3				
	16	12	12	1-2
	16q22-qter	3	3	1-2
	16q23-qter	4	4	1-2
MCF.7				
	16	4	4	1-3
	16q22-qter	2	2	1-2
	16q23-qter	5	5	1-2
	13	3	0	Immortal
Rat				
NMU				
	16	2	2	1-2
	16q22-qter	2	2	1-2
	13	3	0	Immortal
LA7				
	16	15	15	1-2
	16q22-qter	9	9	1-2
	16q23-qter	12	12	1-2

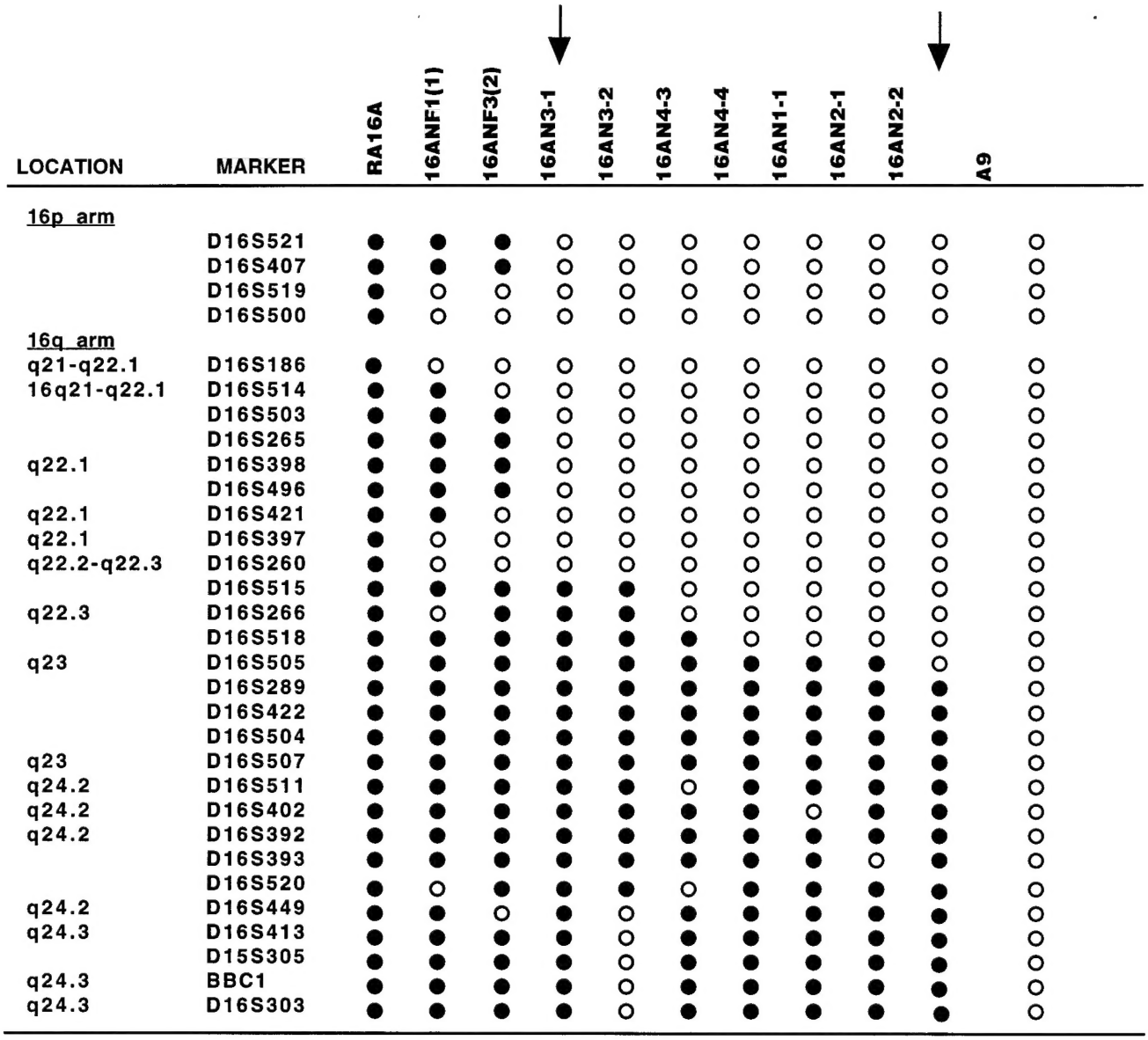

Fig. 1: PCR analysis of chromosome 16 subchromosomal hybrids for chromosome 16 specific markers: Lane 1 represents the intact human chromosome 16, lanes 2-10 represent the fragments of chromosome 16 and lane 11 represents the mouse cell line A9. The position of markers on the human chromosome is indicated in the left most column. Closed circles indicate the presence of the marker while the open circles indicate the absence of the marker on the fragment. The two fragments used for MMCT experiments is indicated by the two arrows.

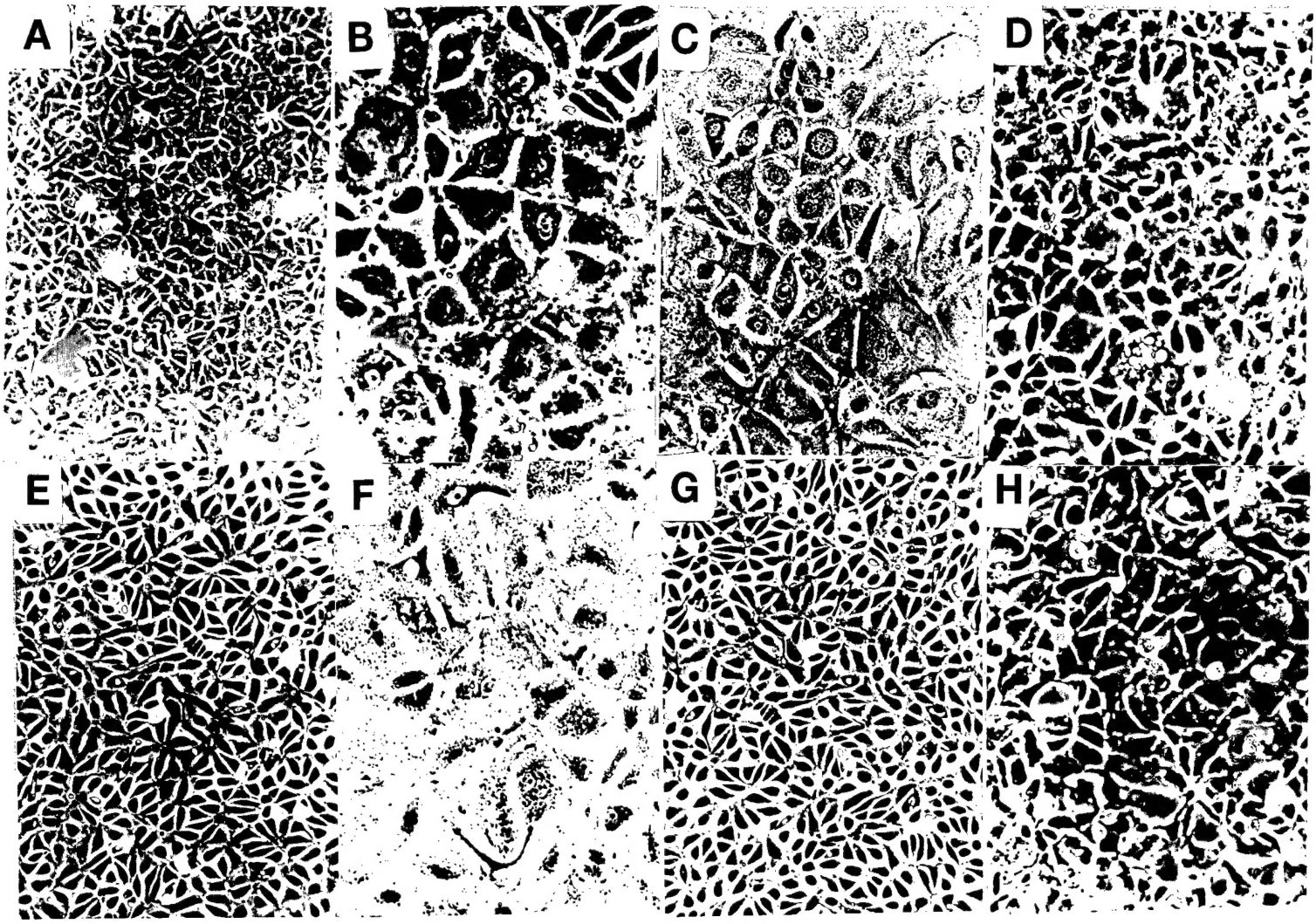

Figure 2: Photomicrographs of human and breast tumor cells before and after the introduction of normal human chromosomes: (**A**) MCF-7, immortal human breast tumor cell line; (**B**) MCF/16, senescent microcell hybrid containing donor chromosome 16; (**C**) MCF/16A2-2, senescent microcell hybrid containing donor chromosome 16q 23-qter; (**D**) MCF-rev, immortal revertant clone from a senescent population of MCF/16; (**E**) LA7, immortal rat mammary tumor cell line; (**F**) LA7/16A2-2, senescent microcell hybrid containing donor chromosome 16A2-2; (**G**) LA7-rev, immortal revertant clone from a senescent population of LA7/16A2-2; (**H**) MCF/13, immortal microcell hybrid containing donor chromosome 13

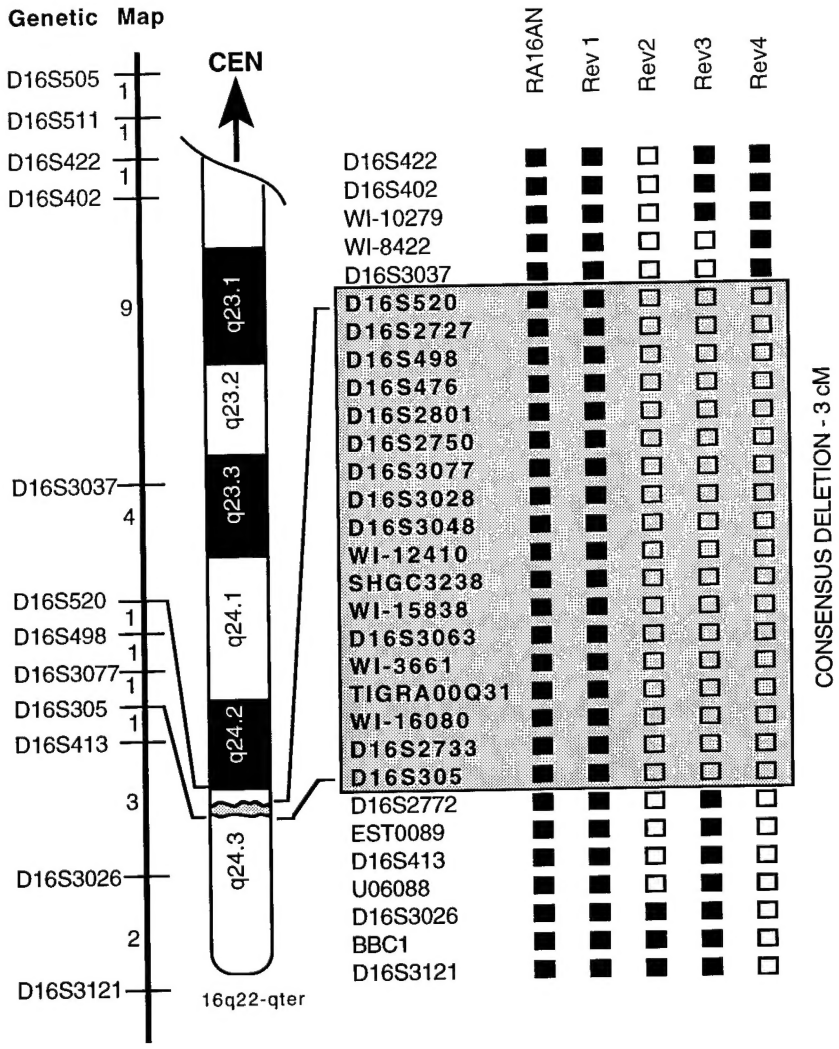

Figure 3: Physical and genetic map of the 16q22-qter region of human chromosome 16. Location of the consensus deletion (boxed) identified from analysis of 35 immortal revertants of the rat mammary tumor cell lines, LA7 and NMU, with 119 chromosome 16 specific markers is indicated. Markers found to be present (closed boxes) or absent (open squares) are indicated in the respective lanes. The first lane represents the introduced nomal human chromosome 16/fragment of chromosome 16 containing 16q22-qter and q23-qter. Rev1 represents PCR data from 28 rat revertants which were positive for all the markers analyzed. Rev 2 and Rev4 represent a mixed population of revertants whereas Rev 3 is a subclone of a mixed population.

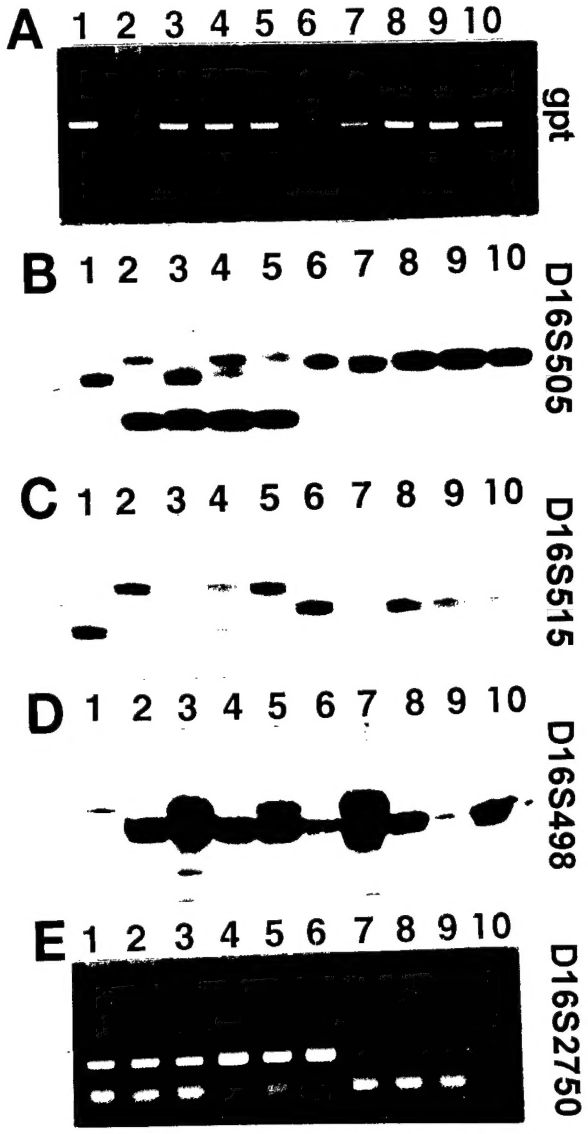

Fig. 4. Analysis of microcell hybrids for donor chromosome 16 specific markers: RA16, donor mouse/human monochromosomal hybrid containing intact 16 (lane 1), MCF-7 (lane 2), MCF-7/16, senescent hybrid of MCF-7 carrying intact 16 (lane 3), MCF-Rev1, revertant clone from a senescent hybrid (lane 4), MCF-Rev2, revertant clone from a different senescent hybrid (lane 5), SKBR-3 (lane 6), SKBR/16, senescent hybrif of SKBR-3 carrying intact 16 (lane 7), SKBR-Rev1, revertant clone from a senescent hybrid (lane 8), SKBR-Rev2, revertant from another senescent clone (lane 9), SKBR-Rev3, another revertant clone from a different senescent hybrid (lane 10). PCR amplification of a 787 bp *gpt* sequence (A), analysis ofor polymorphic dinucleotide (CA)n repeats for loci D16S 505 (B), D16S515 (C) and D16S498(D). Molecular analysis of immortal revertants of senescing microcell hybrids of rat breast cancer cells for D16S2750 (E). RA16, (lane 1), 16A3-1, donor mouse/human monochromosomal hybrid containing fragment of chromosome 16q 22-qter (lane 2), 16A2-2, donor mouse/human monochromosomal hybrid containing fragment of chromosome 16q 23-qter (lane 3), LA7/16, senescent rat breast tumor cells containing intact chromosome 16 (lane 4), LA7/16A3-1, senescent rat tumor cells containing chromosome 16q 22-qter (lane5), LA7/16A2-2, senescent rat tumor cells containing chromosome 16q 23-qter (lane 6), Rev1 (lane 7) and Rev2 (lane 8), revertant clones from senescent clones containing chromosome fragment16A3-1, and, Rev3 (lane 9), revertant clone from senescent clone containing chromosome fragment 16A2-2, all three showing the absence of the marker D16S2750, LA7, rat mammary tumor cell line (lane 10).